DNA copy number profiling of gastric cancer and its clinical implications

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Chapter 13

Summary and general discussion

13.1 Summary

Although incidence rates of gastric cancer are declining, it still remains the second leading cause of cancer death worldwide.[1] The only possible curative treatment is complete surgical resection. Symptoms of gastric cancer are frequently absent until the disease reaches an advanced stage, restricting the chance of curation by surgical treatment.[2] One of the main factors contributing to higher risk of gastric cancer is infection with *H. pylori*, which leads to chronic active gastritis, in turn leading to mucosal atrophy, intestinal metaplasia followed by intraepithelial neoplasia and ultimately will lead to an invasive carcinoma.[3] Besides infection with *H. pylori*, several hereditary, dietary and environmental factors have been described to influence gastric cancer risk.

Gastric cancers often show chromosomal instability, resulting in gains and losses (also referred to as DNA copy number aberrations) of parts of or even whole chromosomes. Gastric cancer is a heterogeneous disease at the genomic level and different patterns of DNA copy number aberrations can reflect differences in clinical behavior and patient outcome.[4,5] Array comparative genomic hybridization (array CGH) is a powerful technique to study profiles of DNA copy number aberrations in cancers. One of the major limitations for using array CGH remains the high costs. Since DNA derived from formalin-fixed and paraffin-embedded (FFPE) samples can be of suboptimal quality and therefore unsuitable for obtaining high quality array CGH results, experimental costs can be reduced by selecting DNAs of which quality is suitable for array CGH analysis. In *chapter 2* we described that isothermal amplification is a reliable predictor for identifying genomic DNAs that are suitable for good quality array CGH outcome.[6] In *chapter 3* we described a method to circumvent reference channel hybridization in every experiment, thereby reducing experimental costs. We showed the possibility of hybridizing two test samples on one array and that a reference channel of a different array can be used for obtaining DNA copy number profiles. We also showed that this approach can be used to distinguish copy number aberrations (CNAs) and copy number variations (CNVs), which have become increasingly relevant because they are easily detected with the current high resolution oligonucleotide based array CGH platforms, in contrast to e.g. the BAC array platforms used until recently.[7]

In *chapter 4* we combined DNA copy number analysis with microarray expression analysis, in order to discover new putative tumor suppressor genes involved in gastric cancer. Using siRNAs directed against *UPF1*, one of the key regulators of the nonsense-mediated decay (NMD) system, we were able to inhibit the NMD mechanism in two gastric cancer cell lines leading to the accumulation of supposedly truncated transcripts. We performed microarray expression analysis by hybridizing cells transfected with siRNA directed against *UPF1* versus cells transfected with non-specific siRNA duplexes (CVII). Integration of the microarray expression and array CGH data provided a list of candidate genes putatively inactivated by nonsense mutation and deletion. We selected 12 genes that only showed one splice variant. We were able to sequence 11 of these genes. Although we did not find mutations leading to premature termination codons, we could detect missense mutations in some of the candidate genes. The biological consequences, if any, of the mutations found still need to be further explored.

To better understand the molecular pathogenesis of gastric cancer, in *chapter 5*, we analyzed gastric cancer precursor lesions, which have the potential to progress to malignancy, by array CGH. We studied two different morphological types of gastric adenomas, i.e. intestinal-type and pyloric gland adenomas, to see if distinct pathways of gastric carcinogenesis would underlie

these two adenoma types. We showed that gains on chromosomes 8, 9q, 11q and 20, and losses on chromosomes 5q, 6, 10 and 13 are early events in gastric carcinogenesis. We also showed that, despite the morphological differences, these two adenoma types are rather similar in terms of DNA copy number aberrations.[8]

There are two main histological types of gastric cancers according to the Laurén classification.[9] It is still controversial if these two histological types differ in terms of DNA copy number aberrations. Some studies describe differences in DNA copy number profiles, while other studies describe similar patterns.[10-13] In addition, a third histological type of gastric cancer has been recognized, showing a dual pattern of differentiation.[14] In *chapter 6* we separately analyzed DNA copy number aberrations of the two different components within the mixed type gastric cancers by array CGH. We found similar patterns of chromosomal aberrations in both histological components within each tumor, showing that the morphological difference is not caused by DNA copy number aberrations.[15]

Gastric cancers mainly occur in the 50-70 year old age group, however still a substantial number of young patients get gastric cancer. In *chapter 7* we aimed to unravel biological differences in gastric cancers from young and elderly patients. We studied gastric cancers of 17 young (<50 years) and 29 elderly (>70 years) patients by array CGH. Hierarchical cluster analysis revealed three clusters with different DNA copy number profiles, significantly correlated with age group. Cluster 1 mainly consisted of young patients and clusters 2 and 3 mainly comprised elderly patients. We showed that the chromosomal regions 11q23.3 and 19p13.3 contributed most to the different genomic profiles of gastric cancers in relation to age of onset. These findings indicated different pathogenic pathways of gastric cancers in young and elderly patients.[16]

In *chapter 8* we investigated if gastric cancers from South African patients showed different patterns of genetic instability compared to gastric cancers from Western European patients. The prevalence of infection with *H. pylori* is high, but incidence of gastric cancer is low in South Africa. This phenomenon is termed the African enigma.[17] Using microsatellite analysis we observed higher frequencies of microsatellite instable gastric cancers in South African patients compared to gastric cancers of Western European patients. Using array CGH and hierarchical cluster analysis, we observed different patterns of DNA copy number aberrations in gastric cancers from South African and Western European patients, indicating different mechanisms of gastric carcinogenesis in gastric cancers from patients of different geographical location.

Next, we investigated if chromosomal aberrations in gastric cancers could be used to identify subgroups of patients that would likely not benefit from intensified therapy, such as extended surgical lymph node dissection or (neo)adjuvant chemo- or radiotherapy. Previous studies, using array CGH and hierarchical cluster analysis, showed significant correlation between cluster membership and lymph node status.[4] In *chapter 9*, we analyzed a large series of 206 gastric cancers by array CGH to obtain an overview of DNA copy number changes in gastric cancers and to unravel chromosomal aberrations correlated to lymph node metastasis en clinical outcome. We found that losses on chromosomes 5q, 10q and 14q were significantly more frequently observed in lymph node negative gastric cancers, and we identified a subgroup of gastric cancers, marked by losses on chromosomes 5q11-q31.3 and 14q32.11-q32.33, that have a good clinical outcome after surgery alone.

In *chapter 10*, we analyzed DNA copy number profiles of 63 gastric cancers by array CGH and by

Multiplex Ligation-dependent Probe Amplification (MLPA) to detect gene-specific DNA copy number aberrations, which we correlated to clinicopathological data. We showed that gain of chromosome 20 was significantly correlated with histological tumor type and with lymph node metastasis. DNA copy number gain of the gene *ZNF217*, located on 20q13.2, was detected in intestinal and mixed type, but not in diffuse type gastric cancers. DNA copy number gain of *TNFRS6B*, located on 20q13.3, was significantly correlated to lymph node metastasis in gastric cancers.

In chapters 11 and 12 we described prognostic markers for gastric cancer patients. In *chapter 11* we used DNA ploidy status to predict prognosis of gastric cancer patients. We analyzed 221 gastric cancers by flow cytometry and image cytometry. We showed that DNA ploidy status obtained by image cytometry is superior in predicting prognosis compared to DNA ploidy status obtained by flow cytometry. In addition, we found that DNA ploidy status in combination with TNM (tumor-node-metastasis) classification is better in predicting clinical outcome compared to both measures alone. In *chapter 12* we showed that promoter hypermethylation status of *MAL* can be used as prognostic marker. Moreover, we showed that promoter hypermethylation correlated with mRNA downregulation indicating that *MAL* is a putative tumor suppressor gene involved in gastric cancer.

13.2 Conclusions and future perspectives

Biological mechanisms and profiling of gastric cancer

Knowledge about the molecular pathogenesis of gastric cancer is still limited. More insight in the biological mechanisms underlying gastric carcinogenesis is important for the development of preventive strategies, for finding markers for early detection, for better classification of gastric cancers in relation to choice of therapy and for finding new therapeutic targets in order to reduce gastric cancer mortality.

Chromosomal instability is one of the hallmarks of solid tumors, including gastric cancer, and results in gains and losses of parts of or even whole chromosomes. Microarray comparative genomic hybridization (array CGH) is a recently developed powerful technique to study these patterns of DNA copy number aberrations, since genome-wide information can be obtained at high resolution in a single experiment. Furthermore, array CGH can be applied on DNA obtained from formalin-fixed and paraffin-embedded (FFPE) tissue samples. This opens up the rich source of large clinical tissue archives with detailed clinicopathological information and clinical follow-up data. One of the major limitations for using genome-wide array CGH analysis remains the high experimental costs. For this reason we provided, in *chapters 2 and 3*, different strategies to reduce experimental costs, making array CGH more suitable for studying large series of clinical samples. Although array CGH is very suitable for DNA copy number profiling, it does not provide information about aberrations that do not result in DNA copy number changes, such as mutations, rearrangements and loss of heterozygozity (LOH). Additional techniques can be used to study these abnormalities.

The field of high throughput genomics is rapidly developing. Methods that were state of the art at the start of this PhD project, like BAC arrays, have been replaced by new platforms like oligonucleotide based array CGH.[18,19] The fact that the large scale studies initiated in this PhD project were performed in BAC arrays while at the same time methodological innovations in the use of oligonucleotide based array CGH were studied, illustrates the situation. New versions of array CGH platforms are emerging with a speed comparable to what was seen for e.g. personal

computers in the 90's of last century.

The latest genome-wide technology in the field of genomics includes massively parallel sequencing (MPS). This technique offers the possibility to analyze the entire genome for DNA copy number changes, point mutations, insertions, deletions and rearrangements in one single experiment. Cancer cell lines have already been analyzed using this technique, but studies are still limited. The limitations posed by experimental costs exist for MPS even more extreme than for array CGH. In the near future, this technique will probably also be generally applied on cancer tissues, providing a whole new spectrum of biological knowledge of the cancer genome.[20,21]

Gastric cancer is the result of interplay between genetic and epigenetic changes. Genetic changes, which can be reflected by chromosomal gains and losses, may lead to the activation of oncogenes and the inactivation of tumor suppressor genes, in turn leading to gastric cancer development and progression. Since there is a large variation in gastric cancer patients with respect to tumor type and genetic and environmental factors, gastric cancers in different patients do not necessarily have to develop by the exact same biological pathways. Patterns of chromosomal aberrations in tumors may reflect different subgroups of cancers that arise through different molecular mechanisms. In addition, certain patterns of chromosomal instability can be correlated to clinicopathological characteristics and disease outcome. In this thesis, patterns of chromosomal aberrations could be significantly correlated to age and origin of the gastric cancer patients. In addition, we could already detect DNA copy number aberrations in gastric cancer precursor lesions by array CGH, indicating that the underlying events probably occur early in gastric cancer carcinogenesis.

With BAC array CGH still large genomic regions of gains and losses are usually detected, making it difficult to pinpoint individual genes contributing to the development or progression of gastric cancer. A first step towards pinpointing candidate genes is described in *chapter 10 and 12*. Using MLPA analysis, DNA copy number gains of specific genes were more frequently observed within a region of chromosomal gain compared to neighboring genes and DNA copy number gains of specific genes could be correlated to clinicopathological data. The next step is to study if gene specific DNA copy number changes have a biological impact at the mRNA or protein expression level.

Another approach in unraveling putative tumor suppressor genes involved in gastric cancer is by studying epigenetic changes, such as promoter hypermethylation. We showed that promoter hypermethylation of the gene *MAL* correlates with reduced mRNA expression. To further evaluate the biological role of putative oncogenes and tumor suppressor genes functional studies are needed. Currently, we are performing functional studies on the *MAL* gene in gastric cancer cell lines. By treating gastric cancer cells with 5-aza-2'-deoxycytidine we expect that the *MAL* gene will be demethylated and consequently overexpressed, proving in that way that silencing of the gene is due to its promoter hypermethylation. Moreover studies are being initiated to transfect gastric cancer cell lines with a vector containing the *MAL* gene to evaluate if overexpression of *MAL* impairs the ability of anchorage-independent growth and migration and invasion of gastric cancer cells. Eventually, to prove the function of *MAL* as tumor suppressor gene in vivo we will inject MAL stably transfected gastric cancer cells into mice to in order to evaluate if these cells lose the ability of forming tumors.

Clinical implications

Secondary prevention can be used as an approach to reduce deaths from gastric cancer. In countries with high prevalence, like Japan, endoscopic screening has been undertaken to reduce mortality rates. In other countries, including the Netherlands, incidence rates of gastric cancers are lower

and therefore population based screening programs are not in place. Strategies to reduce gastric cancer death mainly focus on improving therapeutic strategies, including surgery and (neo)adjuvant chemo- or chemoradiotherapy.

In the Netherlands, surgical treatment generally includes limited (D1) lymph node dissection due to higher morbidity and mortality associated with extended (D2) lymph node dissections. D2 dissection can be beneficial for a subgroup of patients with regional lymph node metastasis. On the other hand, patients with gastric cancers without lymph node metastasis will not benefit from a D2 dissection and are unnecessarily subjected to higher risk of morbidity and mortality. Also subgroups of gastric cancer patients with favorable outcome will hardly benefit from intensified therapy, and will probably rather experience more harm instead. Identifying subgroups of patients with good clinical outcome can be used to select patients who are unlikely to benefit from additional intensified therapies

Array CGH technology might not be the option of choice for clinical practice, however, and a PCR-based test, such as MLPA, may be more suitable. MLPA needs less amounts of input DNA, is easier to perform and enables detection of gene-specific copy number changes. In *chapter 10*, we already showed that this technique can be used to identify gene-specific DNA copy number aberrations correlating with lymph node status. However, this still needs further optimization. In *chapter 9* we show that losses on chromosomes 5q and 14q are detected at significantly higher frequencies in a subgroup of gastric cancers with an excellent clinical outcome after surgery alone. Clinically it could become highly relevant to identify this, and possibly, other molecular subgroups of gastric cancers and adjust the therapeutic regimen to the tumor biology. In this case, refrain from e.g. intensified chemo- and/or radiotherapy. In *chapters 11 and 12*, we show that DNA ploidy status and promoter hypermethylation status are able to predict prognosis of gastric cancer patients. Combining markers may yield a better prediction of disease outcome and needs further investigation. Using a classifier approach, we should take in consideration that subgroups of patients have a different molecular background resulting in different DNA copy number profiles. As we have shown in *chapter 7 and 8*, young and elderly patients have different patterns of gains and losses, but also patients from different geographical location have different genomic instability patterns. Therefore it may be necessary to optimize classifiers for each subgroup of patients.

The biological information obtained from DNA copy number profiles of gastric cancers can also be used to predict response to therapy. Recently, randomized clinical trials (CRITICS, MAGIC-B) have been started in The Netherlands and in the United Kingdom, in which the role of postoperative chemo-radiation in combination with preoperative chemotherapy, and different chemotherapeutic combinations will be evaluated, respectively. Correlation of DNA copy number profiles to drug response can yield biomarkers which can be used to predict response to therapy. These studies may result in more tailored treatment schemes in the future, and in this way contribute to reduce mortality rates of gastric cancer.

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